



ORIGINAL ARTICLE

Bacterial adhesion to antibiotic-loaded guided tissue regeneration membranes — A scanning electron microscopy study



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KEYWORDS

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Background/Purpose: Bacterial contamination of sites undergoing guided tissue regeneration (GTR) therapy may reduce the efficiency of periodontal regeneration. This study compared bacterial adhesion onto various GTR membranes incorporated with antibiotics.

Methods: Three barrier membranes, including expanded polytetrafluoroethylene (ePTFE) membrane, collagen membrane, and glycolide fiber membrane, were loaded with tetracycline or amoxicillin. The adhesion of *Streptococcus mutans* and *Aggregatibacter actinomycetemcomitans* onto the GTR membranes with or without antibiotics was analyzed using the scanning electron microscopy (SEM) analysis.

Results: The SEM analysis showed no apparent alteration in the physical structure of the membranes loaded with antibiotics. Both *S. mutans* and *A. actinomycetemcomitans* attached best on the collagen membranes, followed by the ePTFE membranes, and then the glycolide fiber membranes without antibiotics. Moreover, higher numbers of bacteria were observed on the fibril areas than on the laminar areas of the ePTFE membranes. The amounts of attached

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bacteria on the GTR membranes increased after longer incubation. Incorporation of tetracycline or amoxicillin greatly reduced the adhesion of *S. mutans* and *A. actinomycetemcomitans* onto all of the GTR membranes examined.

Conclusion: Incorporation of tetracycline or amoxicillin greatly reduced adhesion of *S. mutans* or *A. actinomycetemcomitans* on the ePTFE, glycolide fiber, or collagen membranes. This finding indicates that it is valuable and effective to use the antibiotic-loaded GTR membranes for periodontal regeneration therapy.

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Introduction

Guided tissue regeneration (GTR) therapy is a well-established method of regeneration of periodontal tissue that was lost during periodontal disease.¹ GTR procedures have helped achieve pocket elimination, clinical attachment, and successful and predictable alveolar bone fill in various types of bony defects. However, many factors exist that influence the results of GTR, such as plaque control, residual periodontal infection, and smoking habits.

The etiology of periodontitis is primarily the specific bacteria found in the subgingival plaque. Only a few of the microbiota found in the subgingival biofilm are periodontopathic bacteria, including *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*.² Bacterial contamination of the GTR-based surgical sites represents the most significant factor jeopardizing the treatment outcome, such as the formation of new connective tissue attachment or bone.^{3,4} Microbial contamination could affect the attachment of periodontal ligament cells on the GTR membranes.⁵ Bacterial species,^{6,7} bacterial count,^{6,8} and the area of bacterial contamination present on the GTR membranes⁹ are those etiological factors negatively affecting GTR outcome. At the initial 3 minutes of the GTR procedure,¹⁰ GTR membranes have already been contaminated with various Gram-positive bacteria and periodontal pathogens. Periodontal pathogens frequently adhere to and colonize on various kinds of GTR membranes.^{8,9} It has been shown that clinical attachment is even lost when the bacterial count on the GTR membrane is more than 10^8 .⁸ The bacterial count on GTR membranes is positively associated with gingival recession¹¹ and is negatively associated with clinical attachment gain.^{7,9,12} Effective control of microbial contamination has been recognized as an important issue in the regeneration procedure.

The effects of systemic antibiotics on controlling periodontal pathogens are limited and unpredictable after GTR operation.^{13,14} The topical application with antibiotic solution irrigation, antibiotic gel, antibiotic fiber, or antibiotic ointment has been evaluated for GTR treatment outcomes.^{15–18} Local application of 25% metronidazole gel^{15,16} or weekly topical dressing with minocycline ointment for 2 months¹⁷ have substantial beneficial effects on periodontal regeneration. The antibiotic-loaded GTR membrane is a more effective and controlled delivery system to release antibiotics.¹⁹ Incorporating metronidazole into the collagen membrane fails to achieve more substantial periodontal regeneration, but increases the comfort feeling for patients after GTR surgery.²⁰ Using tetracycline-loaded expanded polytetrafluoroethylene (ePTFE) membranes reduces bacterial contamination and increases clinical attachment.²¹

Tetracycline and amoxicillin are effective antibiotics against most periodontal pathogens.^{22–25} According to previous *in vitro* studies, incorporation of amoxicillin or tetracycline into GTR membranes could enhance the attachment of periodontal ligament cells on bacteria-contaminated membranes,²⁶ and inhibit penetration of *Streptococcus mutans* and *A. actinomycetemcomitans* through GTR membranes.²⁷ *S. mutans*, one of the pioneer pathogens of dental plaque formation,²⁸ and *A. actinomycetemcomitans* were analyzed because both species had strong adherence to GTR membranes.²⁹ This study further investigated the bacterial adhesion dynamics on the GTR membranes impregnated with antibiotics, amoxicillin, and tetracycline, using the scanning electron microscopy (SEM) analysis.

Materials and methods

Bacteria and culture conditions

S. mutans (American Type Culture Collection [ATCC] 25175) was cultured at 37 °C in brain heart infusion (BHI) broth or on a BHI agar plate. *A. actinomycetemcomitans* (ATCC33384) was cultured in BHI broth or on an anaerobic blood agar plate (Becton, Dickinson and Company, Cockeysville, MD, USA) and incubated at 35 °C in an anaerobic chamber with anaerobic gas mixture, 5% H₂, 10% CO₂, and 85% N₂.

Preparation of the antibiotic-loaded GTR membranes

Selected sterile GTR membranes, including nonabsorbable ePTFE (Gore-Tex, W.L. Gore & Associates, Flagstaff, AZ, USA), absorbable type I collagen derived from bovine tendon (BioMend, Sulzer Calcitek, Carlsbad, CA, USA), and absorbable glycolide fiber (Resolut XT, W.L. Gore & Associates) were cut into 8-mm-diameter circles using sterile scissors in a tissue culture hood. The ePTFE membranes were dipped in absolute ethanol containing 5% tridodecylmethylammonium chloride (TDMAC) for 1 minute to enhance the antibiotic adsorption capacity and then air-dried in a tissue culture hood at room temperature for 1 hour.^{26,27} Thirty microliters of 8 mg/mL tetracycline or 8 mg/mL amoxicillin solution were added directly to each membrane sample. After drying at 37 °C for 2 hours, the membranes were stored in a desiccator in the dark at room temperature and were analyzed within 1 day.

Observation of bacterial adhesion onto GTR membranes using SEM

The device used for bacterial adhesion experiments was assembled as described previously.²⁷ Because TDMAC may affect bacterial adhesion on the GTR membranes, the ePTFE membranes treated with 5% TDMAC solution were used as a control. Each round membrane, with or without antibiotics, was positioned over an inner glass tube filled with growth media. The tubes were sealed with silicon O-rings and caps. Each tube was placed into a larger outer bottle. A fresh culture of *S. mutans* or *A. actinomycetemcomitans* [5×10^6 colony-forming units (CFU)] in 48 mL BHI was added to the outer bottle. A device without bacteria also served as a negative control.

After incubation for 3 hours or 8 hours for *S. mutans* and 2 days or 3 days for *A. actinomycetemcomitans*, the outer surface of each GTR membrane was analyzed for bacterial adhesion using SEM.²⁶ The adhesion experiment was performed independently twice. The tested GTR membranes were primarily fixed in 2.5% phosphate-buffered glutaraldehyde (pH 7.3) for 10 hours, followed by secondary fixation in 1% osmium tetroxide (OsO_4) for 2 hours. The specimens were then dehydrated in a graded series of ethanol (50%, 70%, 90%, and 100%), followed by dipping into a mixture of 50% isoamyl acetate and 50% ethanol for 15 minutes, and finally transferred into 100% isoamyl acetate for 15 minutes. After being critical point-dried in CO_2 (HCP-2; Hitachi, Tokyo, Japan) and sputter coated with a thin layer of gold (E-101; Hitachi, Tokyo, Japan), the specimens were observed by SEM (S-2700; Hitachi, Tokyo, Japan) at the $\times 1000$ and $\times 3000$ original magnification at accelerating voltage 20 KV to evaluate the bacterial adhesion to the GTR membranes. Moreover, the non-TDMAC ePTFE, collagen, and glycolide fiber membranes were observed by SEM in order to record the original membrane structure.

Results

The original structure of the GTR membranes was analyzed by SEM. The ePTFE membrane was composed of the fibril (Fig. 1A and B) and the laminar areas (Fig. 1C and D). The structure of the glycolide fiber membrane was fiber cross-like and porous (Fig. 1E and F). The collagen membrane contained a continuous panel-like structure (Fig. 1G and H). The SEM analysis showed no apparent alteration in the physical structure of the membranes loaded with antibiotics (data not shown).

After 3 hours of incubation with *S. mutans*, bacteria were found on the collagen and ePTFE membranes without antibiotics (Fig. 2A–C). The numbers of *S. mutans* adhered on the collagen membranes were higher than those adhered on the ePTFE membranes. Adhesion of *S. mutans* was barely observed on the glycolide fiber membranes without antibiotics (Fig. 2D). After incubation for 8 hours, higher numbers of *S. mutans* on the collagen and ePTFE membranes were evident (Fig. 2E–G). For the ePTFE membranes, higher numbers of *S. mutans* were observed on the fibril areas than on the laminar areas. The adhesion of *S. mutans* was apparently reduced on various membranes loaded with tetracycline after incubation for 3 hours (Fig. 3A–D) or 8

hours (Fig. 3E–H). The quantity of adhered bacteria was also apparently decreased on the membranes loaded with amoxicillin (Fig. 4). Thus, the results demonstrated that the numbers of attached *S. mutans* increased after longer incubation. Moreover, incorporation of antibiotics greatly reduced adhesion of *S. mutans* onto GTR membranes.

After incubation for 48 hours on membranes without antibiotics, *A. actinomycetemcomitans* attached best on the collagen membranes (Fig. 5A), followed by the ePTFE membranes (Fig. 5B and C), and then the glycolide fiber membranes (Fig. 5D). After incubation for 72 hours, the numbers of *A. actinomycetemcomitans* increased on each membrane without antibiotics (Fig. 5E–H). Less bacterial attachment was observed on the glycolide fiber membranes than on the other two membranes. Incorporation of tetracycline (Fig. 6) or amoxicillin (Fig. 7) greatly decreased the bacterial adhesion onto all of the GTR membranes examined after 48 hours or 72 hours of incubation. Differences between tetracycline- or amoxicillin-loaded membranes were not observed. Thus, similar to the results from *S. mutans*, incorporation of antibiotics effectively reduced adhesion of *A. actinomycetemcomitans* onto the GTR membranes.

Discussion

Bacterial contamination of sites undergoing GTR therapy may reduce regenerating efficiency.¹¹ Our previous study showed that penetration of *S. mutans* and *A. actinomycetemcomitans* through amoxicillin- or tetracycline-loaded ePTFE, collagen, or glycolide fiber membranes was delayed and/or reduced.²⁷ This *in vitro* study further demonstrated that the numbers of attached bacteria on various GTR membranes increased after longer incubation. Moreover, there was an apparent decrease in the levels of adhesion by *S. mutans* and *A. actinomycetemcomitans* onto the ePTFE, collagen, or glycolide fiber membranes when tetracycline or amoxicillin was loaded onto the GTR membranes. Reduction of bacterial adhesion on the antibiotics-loaded GTR membranes may be due to the antimicrobial effects of antibiotics, as well as the decreased adhesion activity of the pathogens examined.

S. mutans may adhere through a glucan-mediated interaction.³⁰ The adherence properties of *A. actinomycetemcomitans* may be attributed to autoaggregation and the formation of bundled fibrils.³¹ Both species have strong adherence to GTR membranes.²⁹ The current study demonstrated that both bacteria attached mainly on the fibril structure and less on the laminar area of the ePTFE membranes. A similar observation has been shown for *P. gingivalis*, which is also present mainly on the fibrillar region of the ePTFE membranes.³² In the absence of antibiotics, the current study demonstrated that *S. mutans* and *A. actinomycetemcomitans* attached best on the collagen membranes, followed by the ePTFE membranes pretreated with TDMAC, and then the glycolide fiber membranes. In an *in vitro* study by Wang et al.,²⁹ the amounts of *S. mutans* or *A. actinomycetemcomitans* adhered on the collagen membranes are also significantly higher than on the ePTFE membranes. *S. mutans* has stronger adherence onto the ePTFE and collagen membranes than *A. actinomycetemcomitans*.²⁹ Moreover, it has been shown *in vitro*

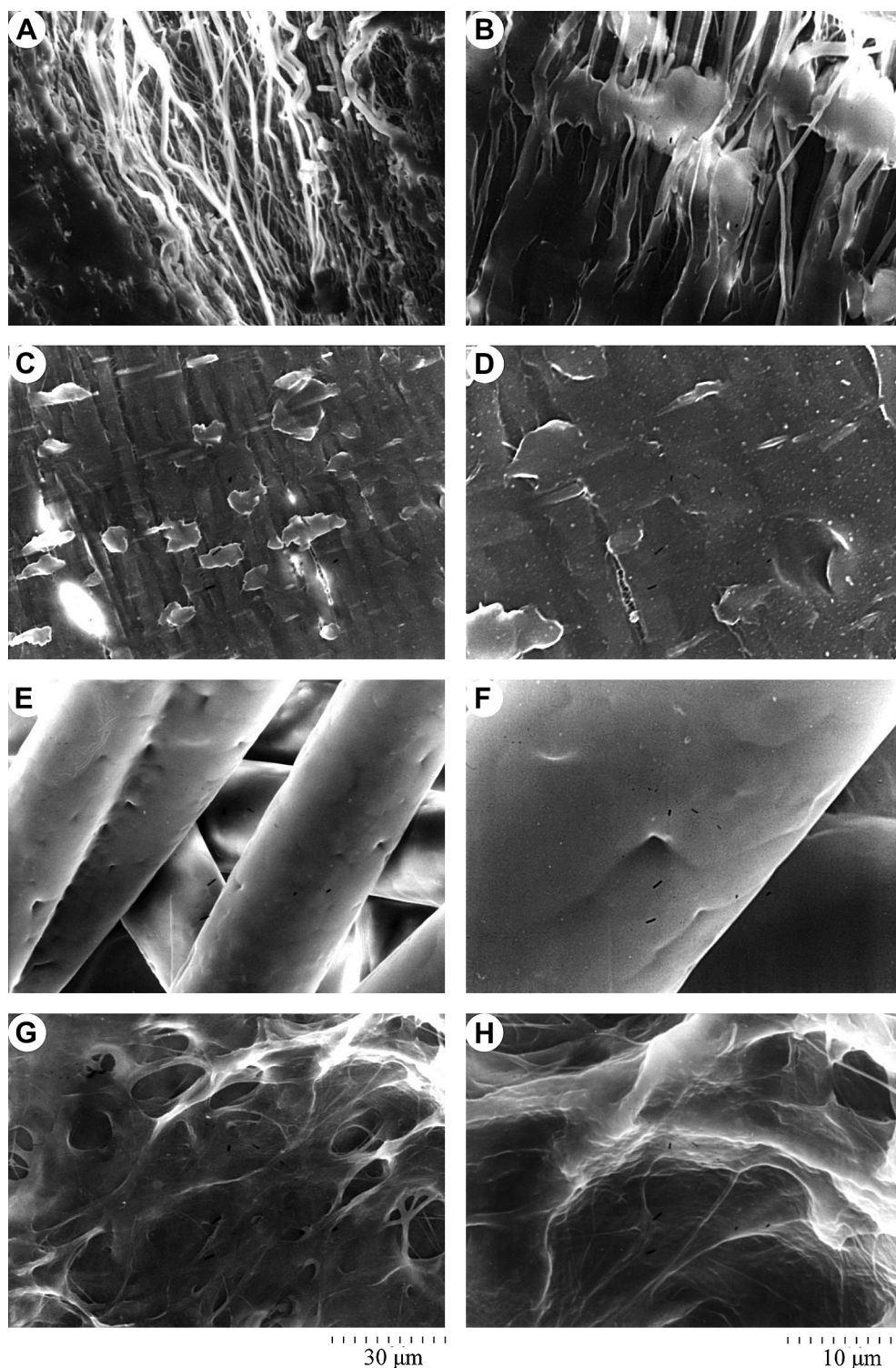


Figure 1 Scanning electron microscopy analysis of the original structure of various guided tissue regeneration membranes. The fibril area (A, B) and the laminar area (C, D) of expanded polytetrafluoroethylene membranes, the fiber cross-like structure of glycolide fiber membranes (E, F) and the panel-like structure of collagen membranes (G, H) were shown (original magnification — panels A, C, E and G: $\times 1,000$; panels B, D, F and H: $\times 3,000$).

that the adherence of *A. actinomycetemcomitans* to collagen is about three times higher than to the ePTFE membranes.³³ It has been reported that bacterial adhesion decreases when the hydrophobicity of biomaterials increases.³⁴ Collagen, which is more hydrophilic than the

other two membranes, might help to create an environment more suitable for bacterial adhesion. Although adhesion of bacteria was barely observed on the glycolide fiber membranes in this study, penetration of *S. mutans* through the glycolide fiber membranes was already evident

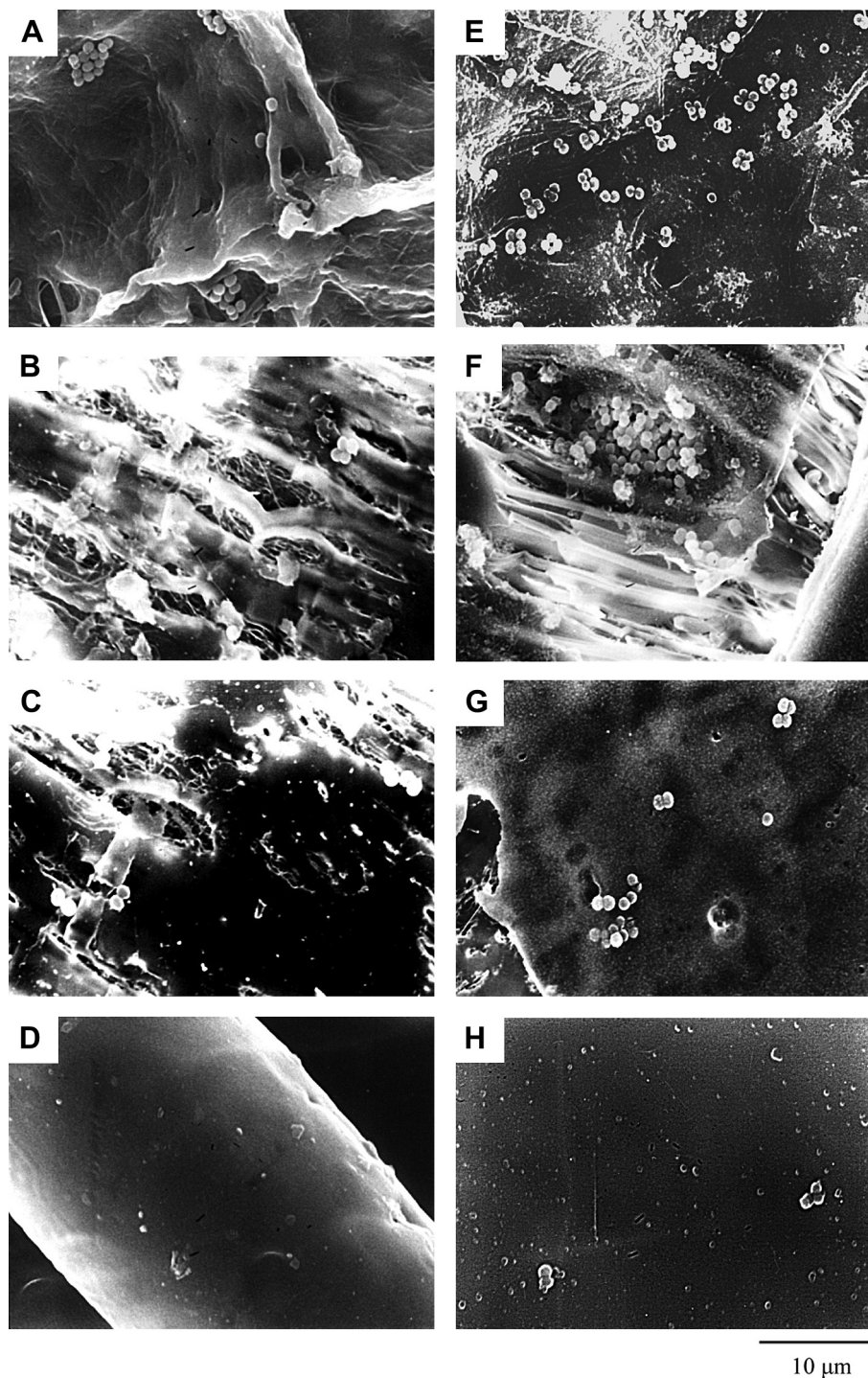


Figure 2 Adhesion of *Streptococcus mutans* on various guided tissue regeneration membranes by scanning electron microscopy (SEM) analysis. *S. mutans* was incubated for 3 hours (A-D) or 8 hours (E-H) on the collagen (A, E), expanded polytetrafluoroethylene (ePTFE, in B, C, F, G) and glycolide fiber (D, H) membranes without antibiotics, followed by SEM analysis. The fibril (B, F) and the laminar (C, G) areas of the ePTFE membrane were shown (original magnification $\times 3,000$).

after 3 hours of incubation,²⁷ indicating that membranes are ineffective at preventing bacterial penetration.

Contamination of GTR membranes with *S. mutans* or *A. actinomycetemcomitans* greatly inhibits the attachment of periodontal ligament cells onto the membranes.⁵ Using tetracycline- or amoxicillin-loaded GTR membranes efficiently reverses the adverse bacterial effects on cellular

attachment *in vitro*.²⁶ The levels of bacterial adhesion on these antibiotics-loaded membranes were also greatly reduced as shown in the current study. The concentration of antibiotics produced in the periodontal pocket by local drug delivery method may be higher than that achieved through systemic administration.³⁵ The release of tetracycline at an antimicrobial concentration can be detected

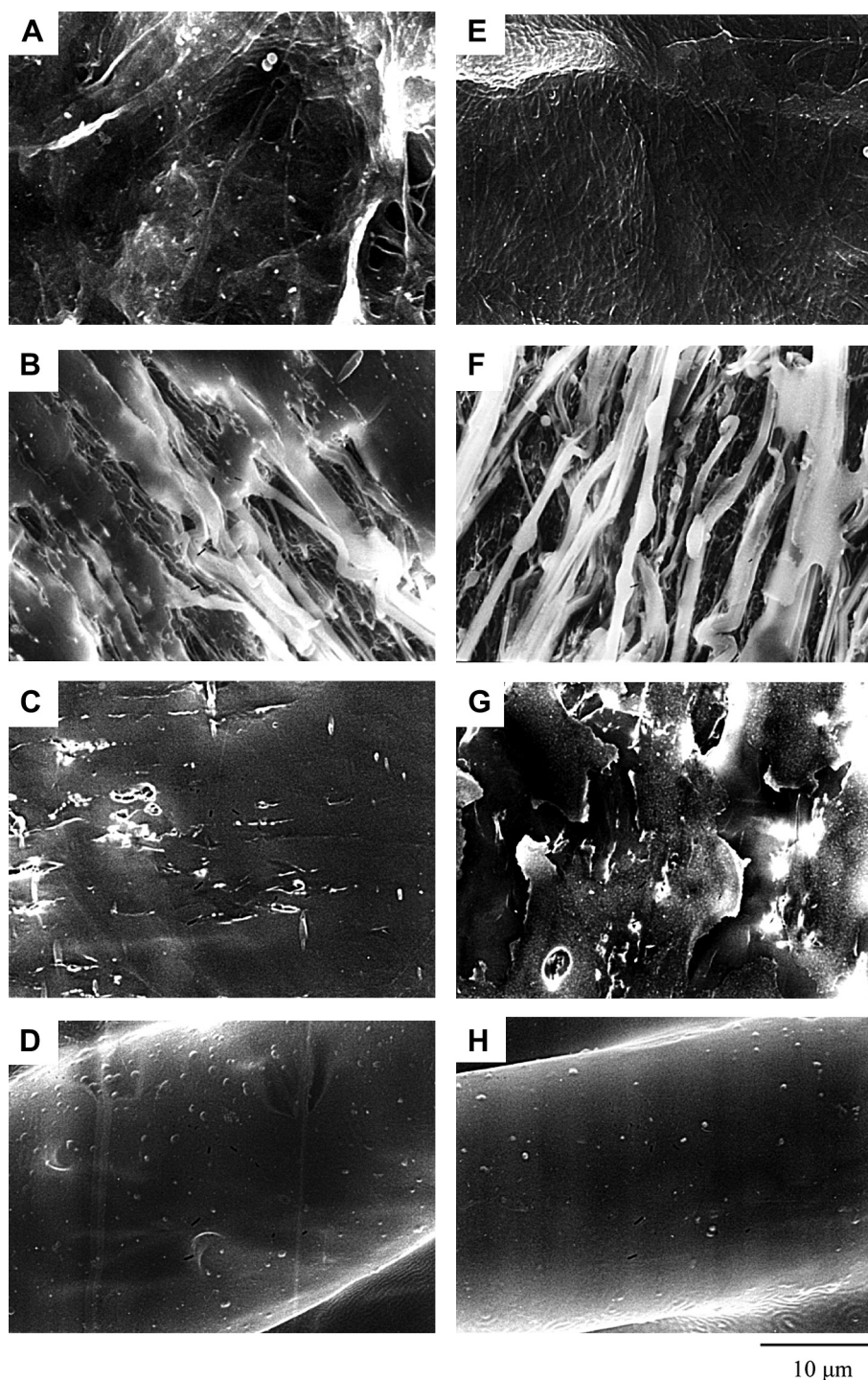


Figure 3 Adhesion of *Streptococcus mutans* on tetracycline-loaded guided tissue regeneration membranes by scanning electron microscopy (SEM) analysis. *S. mutans* was incubated for 3 hours (A-D) or 8 hours (E-H) on the collagen (A, E), expanded polytetrafluoroethylene (ePTFE, in B, C, F, G), and glycolide fiber (D, H) membranes loaded with tetracycline, followed by SEM analysis. The fibril (B, F) and the lamina (C, G) areas of the ePTFE membrane were shown (original magnification $\times 3,000$).

from the impregnated cellulose membranes up to the 12th day.¹⁹ Using tetracycline-loaded ePTFE membranes decreases microbial contamination and increases clinical attachment gain in the treatment of intraosseous defects.²¹ Incorporation of antibiotics onto the GTR membranes may reduce the early bacterial colonization on the GTR membranes and benefit therapeutic outcomes.

The original membrane structure was not affected after incorporation of tetracycline or amoxicillin in our experimental conditions. Incorporation of metronidazole, niridazole, or tinidazole also has no apparent effect on the physical properties of the human type I collagen membranes.³⁶ However, metronidazole may delay the degeneration of the collagen membranes.³⁶ Moreover,

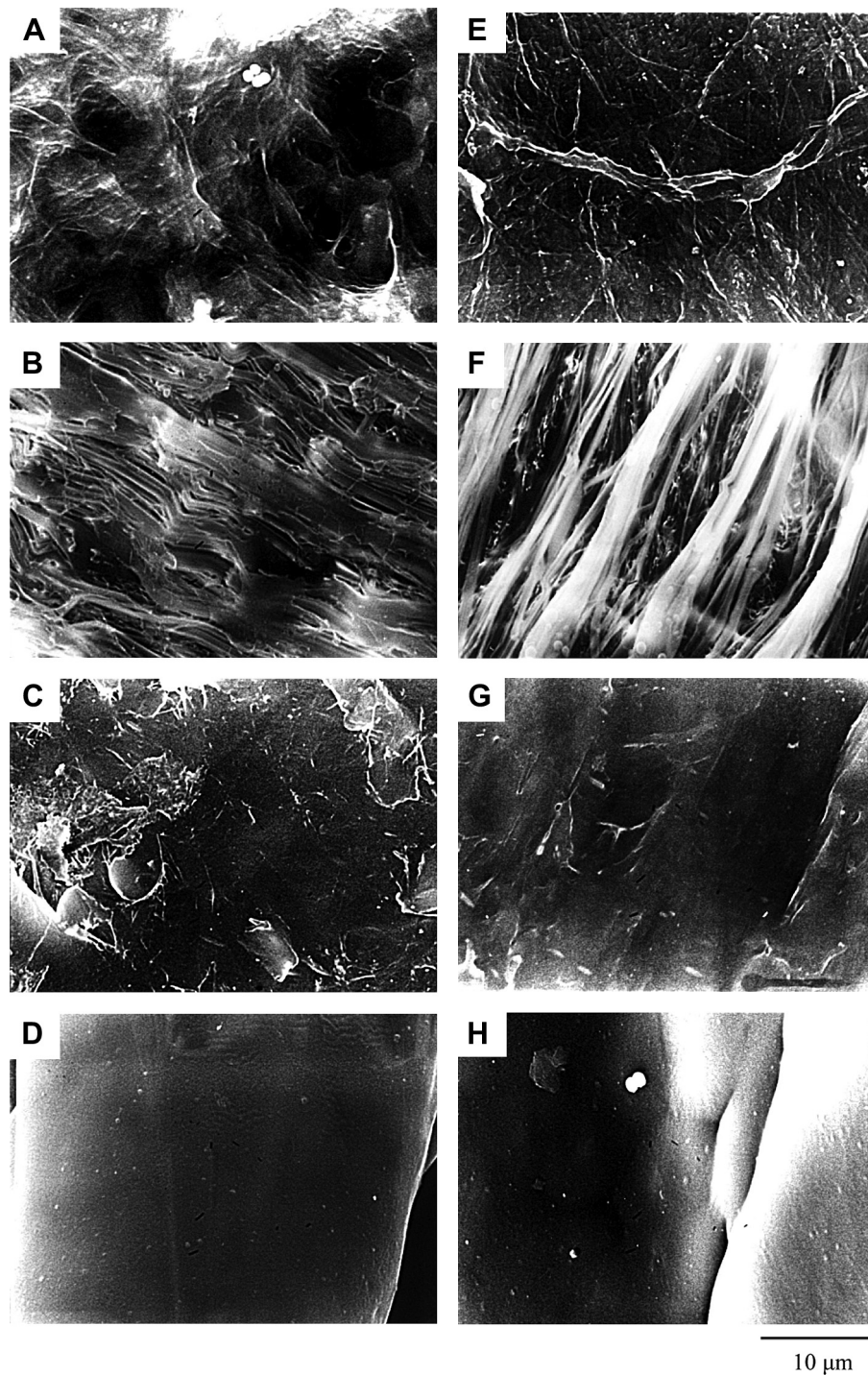


Figure 4 Adhesion of *Streptococcus mutans* on amoxicillin-loaded guided tissue regeneration membranes by scanning electron microscopy (SEM) analysis. *S. mutans* was incubated for 3 hours (A–D) or 8 hours (E–H) on the collagen (A, E), expanded polytetrafluoroethylene (ePTFE, in B, C, F, G) and glycolide fiber (D, H) membranes loaded with amoxicillin, followed by SEM analysis. The fibril (B, F) and the laminar (C, G) areas of the ePTFE membrane were shown (original magnification $\times 3,000$).

tetracycline impregnated on collagen membranes decreases the degradation of collagen *in vitro*³⁷ and *in vivo*.³⁸ Thus, in addition to its antimicrobial activity, tetracycline may also delay degradation and prolong the integrity of collagen membranes to ensure successful cell exclusion.

Penetration of *S. mutans* or *A. actinomycetemcomitans* through tetracycline- or amoxicillin-loaded GTR membranes is significantly decreased and delayed.²⁷ This study further demonstrated that incorporation of tetracycline or amoxicillin significantly decreased adhesion of *S. mutans* or *A. actinomycetemcomitans* onto the ePTFE, collagen, and

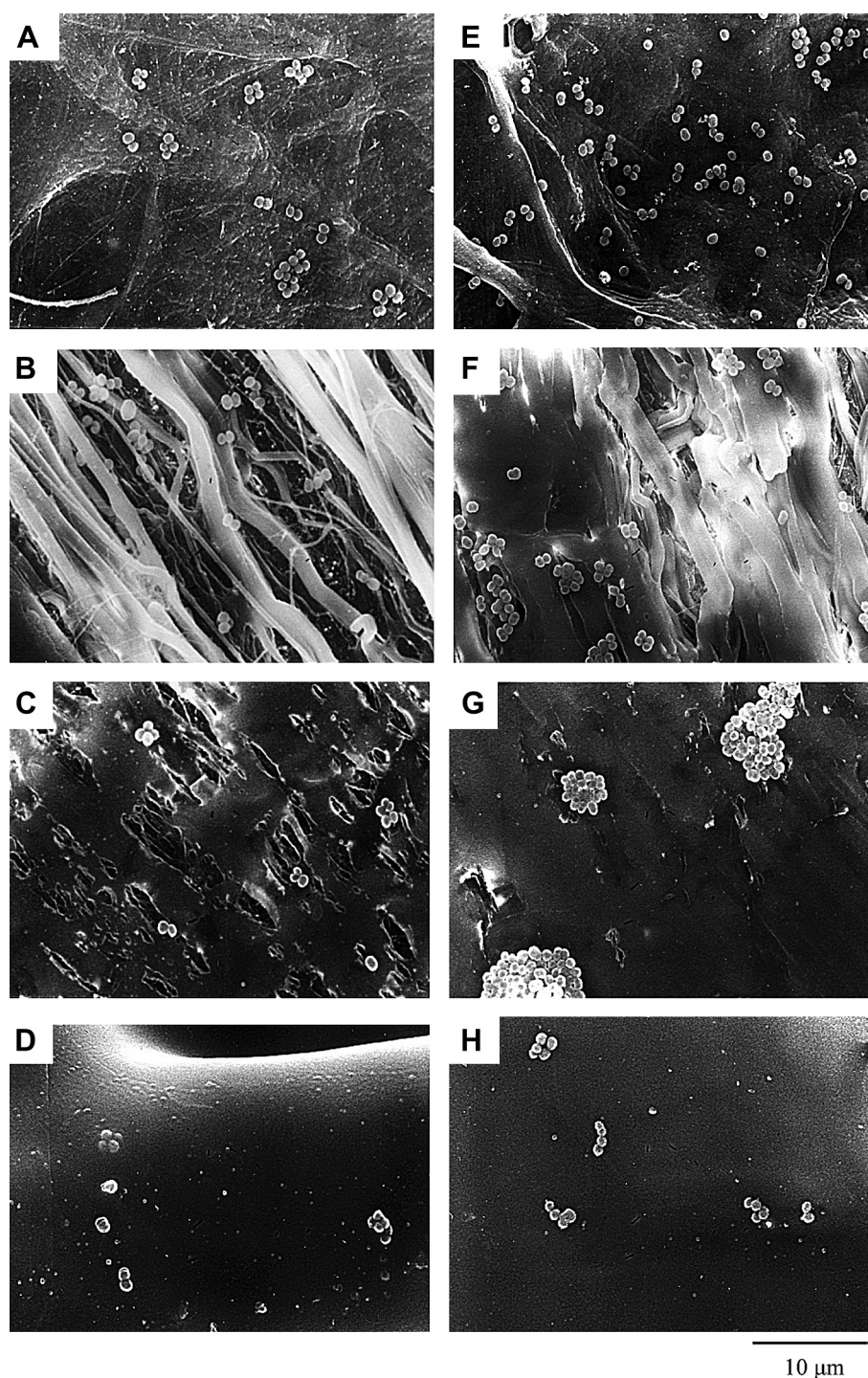


Figure 5 Adhesion of *Aggregatibacter actinomycetemcomitans* on various guided tissue regeneration membranes by scanning electron microscopy (SEM) analysis. A. *actinomycetemcomitans* was incubated for 48 hours (A–D) or 72 hours (E–H) on the collagen (A, E), expanded polytetrafluoroethylene (ePTFE, in B, C, F, G) and glycolide fiber (D, H) membranes without antibiotics, followed by SEM analysis. The fibril (B, F) and the laminar (C, G) areas of the ePTFE membrane were shown (original magnification $\times 3,000$).

glycolide fiber membranes. Different characteristics were also observed among these GTR membranes. The numbers of bacteria on GTR membranes are linked to the gain in probing attachment.⁸ Incorporation of tetracycline or amoxicillin onto these GTR membranes greatly reduced adhesion and penetration of oral pathogens, which is

valuable for the application of the antibiotics-loaded GTR membranes for periodontal regeneration therapy. The clinical effects of tetracycline- or amoxicillin-loaded ePTFE, collagen and glycolide fiber membranes on bacterial dynamic properties and treatment outcomes *in vivo* require further investigation.

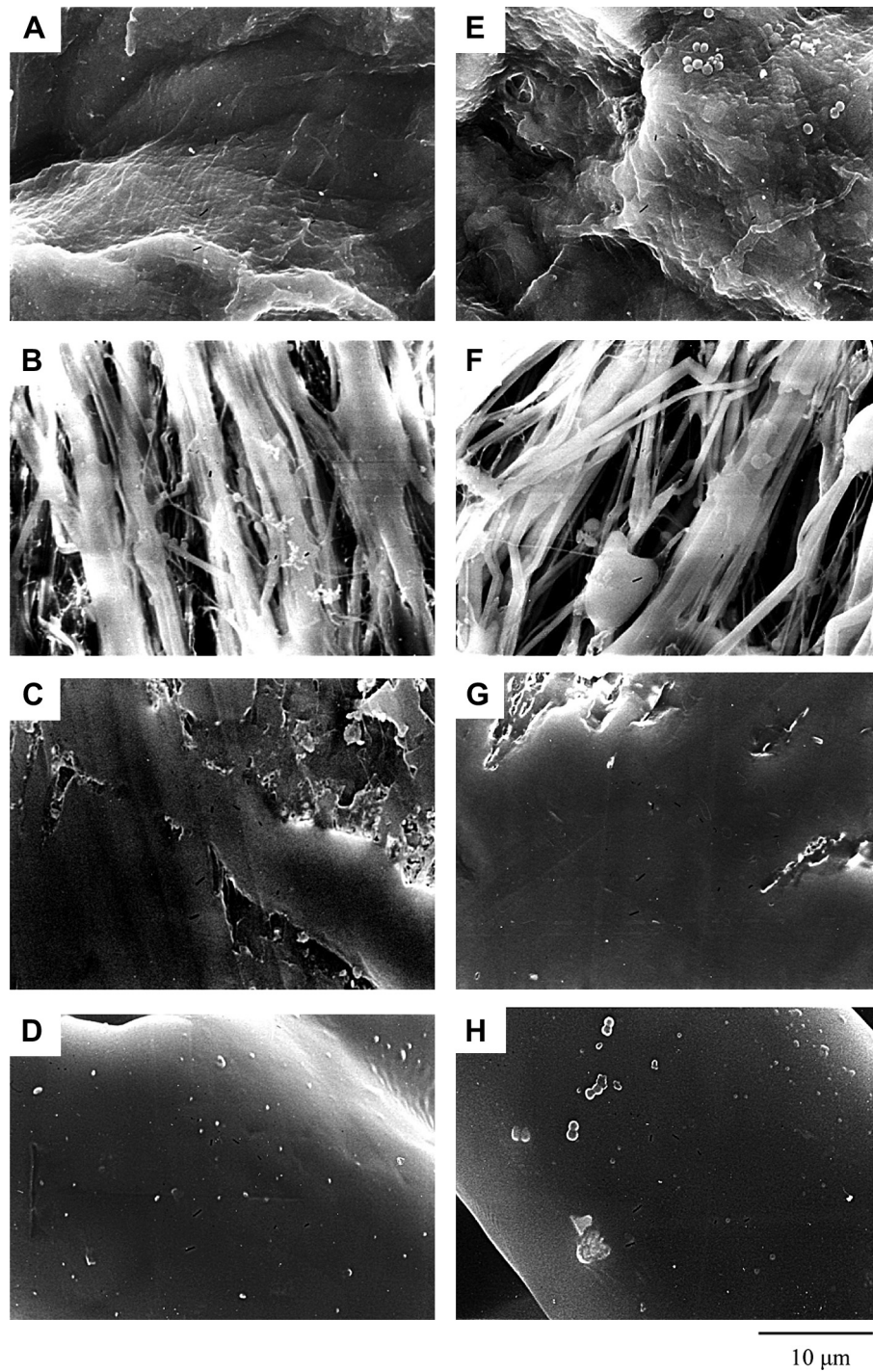


Figure 6 Adhesion of *Aggregatibacter actinomycetemcomitans* on tetracycline-loaded guided tissue regeneration membranes by scanning electron microscopy (SEM) analysis. A. *actinomycetemcomitans* was incubated for 48 hours (A–D) or 72 hours (E–H) on the collagen (A, E), expanded polytetrafluoroethylene (ePTFE, B, C, F, G) and glycolide fiber (D, H) membranes loaded with tetracycline, followed by SEM analysis. The fibril (B, F) and the laminar (C, G) areas of the ePTFE membrane were shown (original magnification $\times 3,000$).

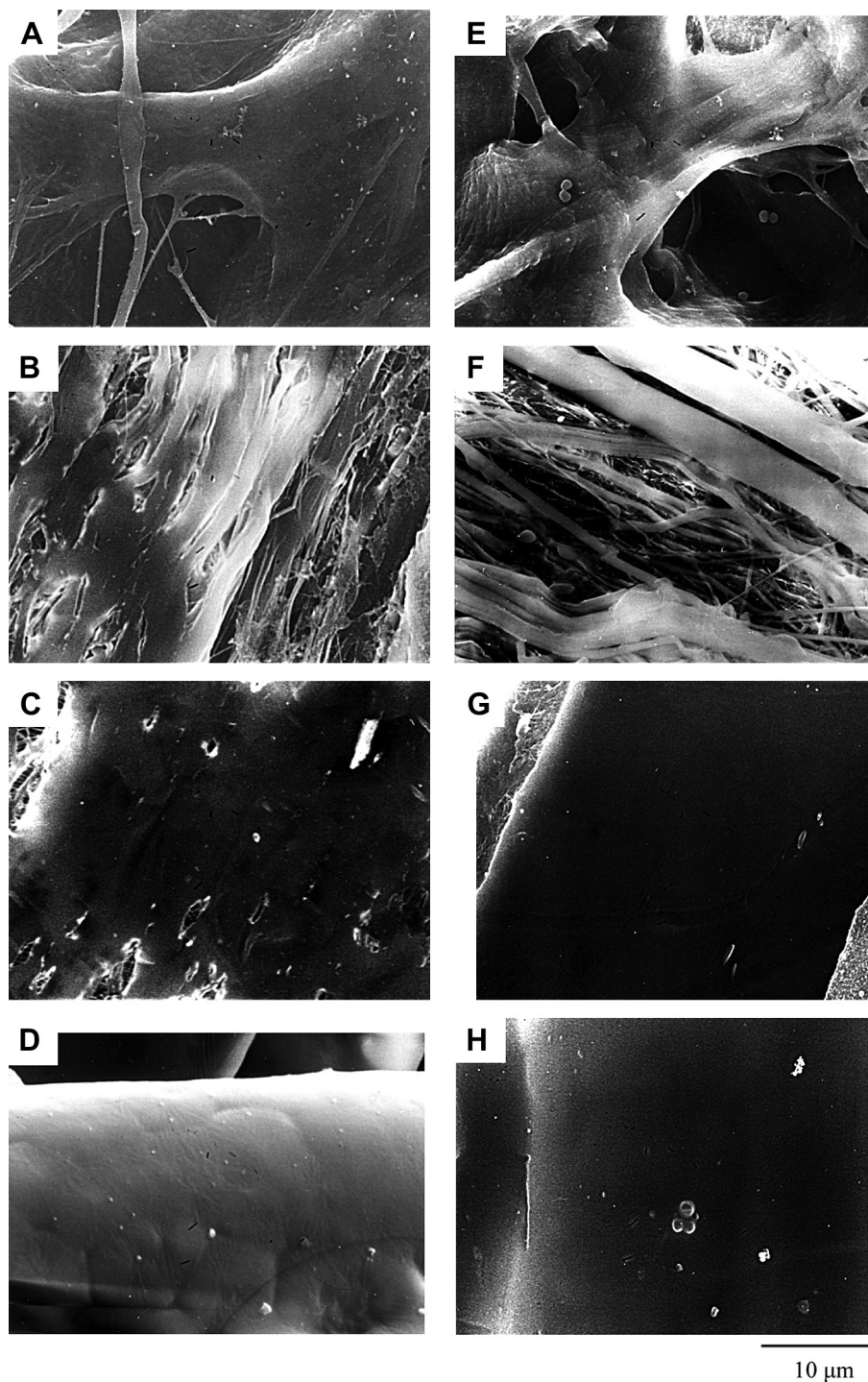


Figure 7 Adhesion of *Aggregatibacter actinomycetemcomitans* on amoxicillin-loaded guided tissue regeneration membranes by scanning electron microscopy (SEM) analysis. A. *actinomycetemcomitans* was incubated for 48 hours (A–D) or 72 hours (E–H) on the collagen (A, E), expanded polytetrafluoroethylene (ePTFE, in B, C, F, G) and glycolide fiber (D, H) membranes loaded with amoxicillin, followed by SEM analysis. The fibril (B, F) and the laminar (C, G) areas of the ePTFE membrane were shown (original magnification $\times 3,000$).

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